IN THE SPECIFICATION

Please amend the specification as follows.

At page 1, amend the title as follows:

~~METHODS AND COMPOUNDS FOR MODULATING NUCLEAR RECEPTOR ACTIVITY~~

COMPLEXES OF ESTROGEN α RECEPTOR BOUND TO AGONIST AND COACTIVATOR MOLECULES

At page 7, paragraph beginning at line 12:

Figure 1 provides a stereo view of the electron density of the complexes, where Figure 1A is a stereo view of the electron density of the DES-ERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) and Figure 1B is a stereo view of the electron density of the OHT-ERα LBD complex (SEQ ID NO: 31). Figure 1 is a graphical representation of a figure that was generated using BOBSCRIPT (Esnouf, *J. Mol. Graph. Model.* 15, 132-4, 112-3 (1997)) and rendered using Raster3D (Merritt, et al., *Acta Crystallogr.* D 50:869-873 (1994)).

At page 7, paragraph beginning at line 18:

Figure 2 was generated using BOBSCRIPT and rendered using Raster3D as described above. Figure 2A shows the overall structure of the DES-ERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) in two orthogonal views. Figure 2B shows the overall structure of the OHT-ERα LBD complex (SEQ ID NO: 31) in two orthogonal views similar to those of the agonist complex in Figure 2A.

At page 8, paragraph beginning at line 24:

Figure 5 was generated using BOBSCRIPT and rendered using Raster3D as described above, and shows a comparison of helix 12 from the OHT complex and the NR Box II peptide. Figure 5A and Figure 5B are stereo views. The structures of the OHT-LBD complex (SEQ ID NO: 31) and the DES-LBD-NR Box II peptide complex (SEQ ID NOs: 27–30) were overlapped

using the Ca coordinates of residues 306-526 of the LBD. Helix 12 from the DES-LBD-coactivator peptide complex is omitted for clarity. Residues 536-551 (helix 12=residues 536-544) from the OHT-LBD complex are colored magenta and the peptide is colored gold. The hydrogen bonds between the a-amino group of Lys 362 and the backbone carbonyls of residues 543 and 544 of helix 12 are illustrated as dashed magenta lines. The hydrogen bonds between the F.-amino group of Lys 362 and the backbone carbonyls of residues 693 and 696 of the coactivator peptide are depicted as dashed orange lines. The following abbreviations are used on helix 12: L540=Leu 540, M543=Met 543, and L544=Leu 544. The following abbreviations are used on the peptide: L690=Leu 690, L693=Leu 693 and L694=Leu 694.

At page 16, paragraph beginning at line 10:

In particular, the present invention relates to the structural and functional effects on the estrogen receptor's LBD, of the binding of two chemically-related compounds, the agonist, diethylstilbestrol (DES), and the selective antagonist 4-hydroxytamoxifen (OHT), the active metabolite of tamoxifen. As described in the Examples, mutagenesis and binding studies, coupled with analysis of atomic models derived from cocrystals, reveals the structure of the human estrogen receptor α ligand binding domain (ERα LBD SEQ ID NOs: 27–30) co-crystallized with a peptide molecule comprising a GRIP I NR Box II peptide sequence (SEQ ID NO:4) (*i.e.*, a peptide derived from the NR Box II region of the p160 coactivator GRIP1) bound to the coactivator binding site and the agonist, DES. Also revealed is the structure of the ERα LBD (SEQ ID NO: 31) co-crystallized with the antagonist, OHT. The Examples provide the 2.03Å resolution crystal structure of the hERα LBD bound to DES and the coactivator and the 1.9Å x-ray crystal structure of the hERα LBD bound to OHT, *i.e.*, the crystals diffract with at least 2.03Å or 1.9Å resolution, respectively.

At page 18, paragraph beginning at line 29:

Chemical modifications will often enhance or reduce interactions between an atom of a LBD amino acid and an atom of a LBD ligand. Steric hindrance will be a common means of

changing the interaction of the LBD binding cavity with the activation domain. Chemical modifications are preferably introduced at C-H, C- and C-OH position in ligands, where the carbon is part of the ligand structure which remains the same after modification is complete. In the case of C-H, C could have 1, 2 or 3 hydrogens, but usually only one hydrogen will be ~~replace.~~ replaced. The H or OH are removed after modification is complete and replaced with the desired chemical moiety.

At page 27, paragraph beginning at line 6:

One method that may be employed for this purpose is molecular replacement. In this method, the unknown crystal structure, may be determined using the structure coordinates of this invention as provided in Appendices 1 and 2. The Appendix 1 coordinates for the DES-ERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) and for the Appendix 2 coordinates for the OHT-ERα LBD complex (SEQ ID NO: 31) have been deposited with the Brookhaven National Laboratory Protein Data Bank, and have been assigned Brookhaven Protein Data Bank Accession Numbers 2erd and 2ert, respectively. This method will provide an accurate structural form for the unknown crystal more quickly and efficiently than attempting to determine such information *ab initio*.

At page 30, paragraph beginning at line 15:

As described above, many coactivators recognize agonist bound nuclear receptor LBDs through the sequence LXXLL (SEQ ID NO:1), where L is leucine and X is any amino acid (the NR box). The structure of the DES-hERα LBD-GRIP1 peptide complex (SEQ ID NOs: 27–30) reveals that the LXXLL motif (SEQ ID NO:1) forms the core of a short amphipathic a helix which is recognized by a highly complementary hydrophobic groove on the surface of the receptor. In agreement with the conclusions of other mutational and structural studies (Brzozowski, *et al.*, *supra* and Feng, *et al.*, supra), it is believed that this peptide binding groove formed by residues from helices 3, 4, 5 and 12 and the turn between helices 3 and 4 is the surface of ERα involved with transcriptional activity, i.e., the coactivator binding site. Further, structural

studies of the complex between TR(3 and the GRIP1 NR box II peptide and biochemical studies of GRIP1 binding to TRβ and GR (Darimont, *et al.*, *supra*) and study of the general features of the PPARγ/SRC-1 peptide complex (Nolte, *et al.*, *supra*) are similar to those of the ERα/GRIP1 NR box II peptide complex described herein, suggesting that the mechanisms of NR box recognition are conserved across the nuclear receptor family.

At Page 34, paragraph beginning at line 3:

Helices 3, 8 and 11 in the DES and E_2 complexes are between one to two turns longer than they are in the OHT complex (Figure 6A and ~~(Brzozowski,~~ Brzozowski, *et al.*, *supra*). Helix 11 ends at Cys 530 in the DES and E_2 complexes and it ends at Tyr 526 in the OHT complex. Helix 12 begins at Leu 536 in the OHT complex. This appears to be necessary; in the antagonist complex, Leu 536 forms a cooperative network of nonpolar contacts and hydrogen bonds with Glu 380 and Tyr 537 that stabilizes the N-terminus of helix 12 (Figure 1B). Therefore, if helix 12 were to bind the static region of the coactivator binding site in the presence of agonist, the loop connecting helices 11 and 12 would be required to span ~17Å over five residues. Although theoretically possible, this conformation would be highly strained and hence unlikely. In contrast, the longer loop connecting helices 11 and 12 in the OHT complex allows helix 12 to extend to the static region of the coactivator binding groove.

At Page 34, paragraph beginning at line 14:

In the DES and E_2 complexes, helix 12 and the loop connecting helices 11 and 12 pack against helices 3 and 11, whereas they do not in the OHT complex (Figures 2A and 2B and ~~(Brzozowski,~~ Brzozowski, *et al.*, *supra*). A recently described structure of the E_2-LBD complex suggests that the longer helices in the DES and E2 complexes are not dependent upon the interactions helix 12 forms in the agonist-bound conformation (Tanenbaum, *et al.*, *supra*). In this structure, a crystal packing artifact forces helix 12 to contact a symmetry-related molecule. Helix 12 is clearly not positioned over the ligand binding pocket in this structure. Nevertheless, helices 3, 8 and 11 are longer than they are in the OHT complex (Figure 6A). Hence the longer

helices of the agonist complexes occur independently of the positioning of helix 12 over the ligand binding pocket and are instead a direct result of agonist binding.

At Page 37, paragraph beginning at line 4:

Crystals of the DES-hERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) were obtained by hanging drop vapor diffusion. Prior to crystallization, the DES-hERα LBD (residues 297-554) complex was incubated with a 2-4 fold molar excess of the GRIP1 NR Box II peptide (SEQ ID NO:4) for 7-16 hr. Two μL samples of this solution were mixed with equal volume samples of reservoir buffer consisting of 25-27% (w/v) PEG 4000, 90 mM Tris (pH 8.75-9.0) and 180 mM Na Acetate and suspended over wells containing 800 μL of the reservoir buffer. After 4-7 days at 19-21°C, rod-like crystals were obtained. The coactivator complex crystals lie in the spacegroup P21 with cell dimensions a=54.09, b=82.22, c=58.04 and 0=111.34. Two molecules each of the DES-LBD and the coactivator peptide form the asymmetric unit. A 200 μm x 40 μm x 40 μm crystal was transferred to a cryosolvent solution containing 25% (w/v) PEG 4000, 10% (w/v) ethylene glycol, 100 mM Tris (pH 8.5), 200 mM Na Acetate and 10 μM peptide and frozen in an N_2 stream at –170°C in a rayon loop. Diffraction data from this crystal were measured at –170°C using a 300 mm MAR image plate at the Stanford Synchrotron Radiation Laboratory (SSRL) at beamline 7-1 at a wavelength of 1.08Å.

At Page 37, paragraph beginning at line 18:

Crystals of the hERα LBD (SEQ ID NO: 31) complexed to OHT were obtained by the hanging drop vapor diffusion method. Equal volume aliquots (2 μL) of a solution containing 3.9 mg/mL protein¬ligand complex and the reservoir solution containing 9% (w/v) PEG 8000, 6% (w/v) ethylene glycol, 50 mM HEPES (pH 6.7) and 200 mM NaCl were mixed and suspended over 800 μL of the reservoir solution. Hexagonal plate-like crystals formed after 4-7 days at 21-23°C. Both crystal size and quality were improved through microseeding techniques. These crystals belong to the space group P6522 with cell parameters a=b=58.24Å and c=277.47Å. The asymmetric unit consists of a single LBD monomer; the dimer axis lies along a crystallographic

two-fold. A single crystal (400 μm x 250 μm x 40 μm) was briefly incubated in a cryoprotectant solution consisting of 10% (w/v) PEG 8000, 25% (w/v) ethylene glycol, 50 mM HEPES (pH 7.0) and 200 mM NaCl and then flash frozen in liquid N_2 suspended in a rayon loop. Diffraction data were measured at -170°C using a 345 mm MAR image plate at SSRL at beamline 9-1 and at a wavelength of 0.98Å.

At Page 39 paragraph beginning at line 10

The high resolution data set of the DES-LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) became available when the R_{free} of the OHT-LBD model was ~31%. Both monomers in the asymmetric unit of the DES complex crystal were relocated using AMoRe and the incompletely refined OHT-LBD model (with helix 12 and the loop between helices 11 and 12 removed) as the search model. The missing parts of the model were built and the rest of the model was corrected using MOLOC and two-fold averaged maps generated in DM. Initially, refinement was carried out with REFMAC using tight NCS restraints. At later stages, the model was refined without NCS restraints using the simulated annealing, positional and B-factor refinement protocols in X-PLOR and a maximum-likelihood target. All B-factors were refined isotropically and anisotropic scaling and a bulk solvent correction were used. The R_{free} set contained a random sample of 6.5% of all data. In refinement, all data between 27 and 2.03Å (with no a cutoff) were used. The final model was composed of residues 305-549 of monomer A, residues 305-461 and 470-554 of monomer B, residues 687-697 of peptide A, residues 686-696 of peptide B, two ligand molecules, 147 waters, two carboxymethyl groups and a chloride ion. According to PROCHECK, 93.7% of all residues in the model were in the core regions of the Ramachandran plot and none were in the disallowed regions.

At Page 40 paragraph beginning at line 21

An electrophoretic mobility shift assay was used to demonstrate that the GRIP1 NR Box II peptide (SEQ ID NO:4) bound the ERα LBD in the presence of the agonist, DES, but not the antagonist, OHT. Eight microgram samples of purified hERα-LBD bound to either DES or OHT

were incubated in the absence of the GRIP I NR Box II peptide (SEQ ID NO:4), i.e., buffer alone, or in the presence of either a 2-fold or 10-fold molar excess of the GRIP1 NR Box II peptide (SEQ ID NO:4). The binding reactions were performed on ice for 45 minutes in 10 µl of buffer containing 20mM Tris, pH 8.1, 1mM DTT, and 200mM NaCl and then subjected to 6% native PAGE. Gels were stained with GELCODE Blue Stain reagent (Pierce).

At Page 40 paragraph beginning at line 29

In the presence of the NR box II peptide, the migration of the DES-LBD complex was retarded. In contrast, peptide addition had no effect on the mobility of the OHT-LBD complex. Hence, this peptide fragment of GRIP1 possesses the ligand-dependent receptor binding activity characteristic of the full-length protein. These observations suggest that the GRIP1 NR Box II peptide (SEQ ID NO:4) is a valid model for studying the interaction between GRIP1 and the ERα LBD.

At Page 41 paragraph beginning at line 3

In order to characterize structurally the interaction between the GRIP1 NR Box II peptide (SEQ ID NO:4) and the ERα LBD, recombinant human ERα LBD (residues 297-554) was crystallized bound to both DES and the GRIP1 NR Box II peptide (SEQ ID NO:4). The ERα LBD bound to OHT was also crystallized in order to determine the mechanism by which this antagonist blocks coactivator/ERα interaction. X-ray diffraction data from these crystals were measured and the structures were determined by a combination of molecular replacement (using a modified version of the coordinates of the human retinoic acid receptor γ (RARγ) LBD, Renaud, *et al.*, *supra*, as the search model) and aggressive density modification.

At Page 41 paragraph beginning at line 11, and subsequent table, footer and heading

The structure of the DES-ERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) has been refined to a crystallographic R-factor of 19.9% (R_{free}=25.0%) using data to 2.03Å resolution, as shown in Figure 1A and Table 2. The structure of the OHT-ERα LBD

complex (SEQ ID NO: 31) has been refined using data to 1.90Å to a crystallographic R-factor of 23.0% (R_{free}=26.2%), as shown in Figure 1B and Table 2.

Table 2
Summary of Crystallographic Statistics

	Ligand	
Data Collection	DES	OHT
Space group	$P2_1$	$P6_522$
Resolution	2.03	1.90
Observations	104189	269253
Unique	30265	23064
Completeness (%)	98.4	99.1
R_{sym}(%)[a]	7.8	7.0
Average I/σI	9.8	16.1
Refinement		
Number of non-hydrogen atoms	4180	2070
R_{cryst} (%)[b]/R_{free} (%)	19.9/25.0	23.0/26.1
Bond r.m.s. deviation (Å)	0.006	0.006
Angle r.m.s. deviation (°)	1.05	1.05
Average B factor (Å^2)	34.0	40.4

[a] $R_{sym} = \sum_i | I_i - \langle I_i \rangle | / \sum_i I_i$ where $\langle I_i \rangle$ is the average intensity over symmetry equivalents

[b] $R_{cryst} = \sum | F_o - F_c | / \sum | F_o |$

Example 3

Overall Structure of the DES-LBD-GRIP1 NR Box II Peptide Complex

At Page 42, paragraph beginning at line 3:

The asymmetric unit of the DES-LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) crystals contains the same noncrystallographic dimer of LBDs that has been observed in the previously determined structures of the LBD bound to both E_2 and RAL (Brzozowski, *et al.*,

supra and Tanenbaum, *et al.*, *supra*). Beyond the flexible loops between helices 2 and 3 and helices 9 and 10, the two LBDs of the dimer adopt similar structures (r.m.s.d. 0.47Å based on Cα positions). The conformation of each LBD complexed with DES closely resembles that of the LBD bound to E_2 (Brzozowski, *et al.*, *supra*); each monomer is a wedge shaped molecule consisting of three layers of eleven to twelve helices and a single beta hairpin (Figure 2A). In each LBD, the hydrophobic face of helix 12 is packed against helices 3, 5/6 and 11 covering the ligand binding pocket (Figure 2A). One GRIP1 NR Box II peptide is bound to each LBD in a hydrophobic cleft composed of residues from helices 3, 4, 5 and 12 and the turn between 3 and 4 (Figures 2A and 3A). The density for both peptides in the asymmetric unit is continuous and unambiguous (Figure IA). Residues 687 to 697 from the GRIP1 NR Box II peptide (SEQ ID NO:4), designated peptide A (SEQ ID NO:29), and residues 686 to 696 from the GRIP1 NR Box II peptide (SEQ ID NO:4), designated peptide B (SEQ ID NO:30), have been modeled; the remaining residues are disordered. Given that each peptide lies within a different environment within the crystal, it is striking that from residues Ile 689 to Gln 695 each peptide forms a two turn, amphipathic a helix (Figures 2A and 3A). Flanking this region of common secondary structure, the peptides adopt dissimilar random coil conformations.

At Page 42, paragraph beginning at line 21 and subsequent header:

The overall structures of the DES-ERα LBD-GRIP1 NR Box II peptide complex (SEQ ID NOs: 27–30) and the OHT-ERα LBD complex (SEQ ID NO: 31) are illustrated in Figure 2. In Figure 2A, the coactivator peptide and the LBD are shown as ribbon drawings. The peptide is colored gold and helix 12 (residues 538-546) is colored magenta. Helices 3, 4 and 5 (labeled H3, H4 and H5 respectively) are colored blue. DES, colored green, is shown in space-filling representation. In Figure 2B, the LBD is depicted as a ribbon drawing. As in Figure 2A, helix 12 (residues 536-544) is colored in magenta and helices 3, 4 and 5 are colored blue. OHT, in red, is shown in space-filling representation.

Example 4

The NR Box II Peptide-LBD Interface

At Page 42, paragraph beginning at line 31:

The binding of the GRIP1 NR Box II peptide (SEQ ID NO:4) to the ERα LBD buries 1000Å^2 of predominantly hydrophobic surface area from both molecules. The GRIP1 NR Box II peptide binding site is a shallow groove composed of residues Leu 354, Val 355, Ile 358, Ala 361 and Lys 362 from helix 3; Phe 367 and Val 368 from helix 4; Leu 372 from the turn between helices 3 and 4; Gln 375, Val 376, Leu 379 and Glu 380 from helix 5; and Asp 538, Leu 539, Glu 542 and Met 543 from helix 12 (Figure 3A). The floor and sides of this groove are completely nonpolar, but the ends of this groove are charged (Figure 3C).

At Page 44, paragraph beginning at line 5:

To test the importance of the GRIP1 NR Box II peptide (SEQ ID NO:4)/LBD interface observed in the crystal, a series of site-directed mutations were introduced into the ERα LBD. These mutations were designed either to simultaneously perturb the structural integrity and the nonpolar character of the floor of the binding groove (Ile 358->Arg, Val 376->Arg and Leu 539->Arg) or to prevent the formation of the capping interactions (Lys 362->Ala and Glu 542->Lys). Fusions of glutathione-S-transferase (GST) to the wild-type and mutant LBDs were analyzed for their ability to bind ^{35}S-labeled GRIM in the absence of ligand or in the presence of DES or OHT.

At Page 49, paragraph beginning at line 2:

Atomic Coordinates for a dimer formed from a first complex having a portion of Human ERα (SEQ ID NO: 27~~ and SEQ ID NO: 28~~) Complexed with DES and a GRIP1 NR Box II Peptide (SEQ ID NO: 29~~ and SEQ ID NO: 30~~), and a second complex having a portion of Human ERα (SEQ ID NO: 28) Complexed with DES and a GRIP1 NR Box II Peptide (SEQ ID NO: 30)